Evol Ecol (2014) 28:869–884
DOI 10.1007/s10682-014-9714-y

ORIGINAL PAPER

Stabilizing selection on nectar concentration in wild *Petunia axillaris*, as revealed by genetic analysis of pollen dispersal

Gabriela Gleiser · Antonina Ingrid Internicola · Frédéric Austerlitz · Giorgina Bernasconi

Received: 18 November 2013 / Accepted: 19 May 2014 / Published online: 30 May 2014

Abstract Most animal-pollinated plants produce nectar as a pollinator reward. Despite the main role that nectar plays in plant-pollinator interactions, the impact of natural variation in nectar traits on realized male fitness is poorly known. Here, we assessed this relation for a wild *Petunia axillaris* population using paternity-based direct selection gradient analysis, which allowed us also to infer pollen dispersal patterns. Because male fecundity may depend on other traits which could be associated with nectar characteristics (i.e. volume and concentration), we also considered selection on other key reproductive traits. The analysis revealed that *P. axillaris* was a strict outcrosser, but that successful pollination occurred mainly among neighbours. Individual plants varied greatly in their male fecundity. Nectar concentration, a key feature of nectar that determines its profitability, was subjected to stabilizing selection. Selection through male function also affected corolla area (positive directional selection), corolla tube length (negative directional selection), and floral display size (stabilizing selection), but none of these traits were phenotypically correlated with nectar characteristics. Because nectar concentration affects the ability and foraging efficiency of different flower visitors to feed on nectar, stabilizing selection may reflect either the preference of the most effective pollinators, or antagonistic selection driven by pollinators and non-pollinating nectar consumers.

Electronic supplementary material The online version of this article (doi:10.1007/s10682-014-9714-y) contains supplementary material, which is available to authorized users.

G. Gleiser (✉)
Laboratorio Ecotono, INIBIOMA, CONICET, Universidad del Comahue, 8400 Bariloche, Argentina
e-mail: gabriela.gleiser@comahue-conicet.gob.ar

G. Gleiser · A. I. Internicola · G. Bernasconi
Institute of Biology, Evolutionary Botany, University of Neuchâtel, Rue Emile Argand 11, 2000 Neuchâtel, Switzerland

F. Austerlitz
Laboratoire d' Eco-anthropologie et Ethnobiologie, UMR 7206, CNRS, MNHN, Univ Paris Diderot, Sorbonne Paris Cité, 75231 Paris, France

Keywords Nectar concentration · *Petunia axillaris* · Pollen dispersal · Seed paternity · Selection gradients · Stabilizing selection

Introduction

Natural selection is broadly accepted as a major mechanism driving evolution of reproductive traits. Phenotypic selection occurs when there is a correlation between phenotypic variation and reproductive success and/or survival (Endler 1986). Ever since Lande and Arnold (1983) introduced the selection gradient approach for studying phenotypic selection in natural populations, there has been an explosion of studies on the subject (reviewed in e.g. Harder and Johnson 2009; Kingsolver et al. 2012). In plants, most studies estimating selection gradients in wild populations have been performed on hermaphroditic species, with a historically stronger focus on the easier-to-measure female function (Conner 2006). However, given that hermaphrodite plants obtain, on average, half of their fitness through the male function, accounting for male fitness becomes essential. This is particularly important for understanding selection on floral rewards, which are probably more critical to enhance pollen export than pollen receipt (Bell 1985; Mitchell 1993; Carlson 2007). Specifically, most flowering plants produce nectar as the main reward to pollinators, suggesting that nectar traits may be shaped by selection to increase pollen export. However, the consequences of variation in nectar traits on male fitness under natural pollination have been rarely explored (e.g. Mitchell 1993; Hodges 1995).

Male reproductive success has often been estimated indirectly by measuring pollen removal, recording pollinator visitation rates, or tracking pollen movements with fluorescent dyes (e.g. Nilsson 1988; Campbell 1989; Cuartas-Domínguez and Medel 2010). However, these measures may be less robust predictors of realized reproductive success (Snow and Lewis 1993; Klinkhamer et al. 1994; Conner 2006) than male fitness estimates obtained combining the use of highly variable molecular markers together with statistical techniques of paternity assignment (e.g. Meagher 1986; Smouse and Meagher 1994; Wright and Meagher 2004; Hodgins and Barrett 2008). Since typically seeds or seedlings are genotyped, such genetically based paternity analyses estimate the realized siring success of pollen donors resulting from both pre- and post-zygotic processes (e.g. Bernasconi 2003; Harder and Routley 2006; Lankinen et al. 2006; Teixeira et al. 2008). Paternity analyses can then be applied to estimate male selection gradients, either indirectly, by regressing estimated individual male fertilities on measured phenotypic variables (e.g. Conner et al. 1996; van Kleunen and Ritland 2004; Kulbaba and Worley 2012), or directly, with maximum-likelihood methods that infer the selection coefficients from adult and offspring genotypes and phenotypes (e.g. Smouse et al. 1999; Morgan and Conner 2001; van Kleunen and Burczyk 2008). Direct estimation of selection gradients provides higher statistical power than indirect estimation, which suffers from inflated error variance arising from the fact that male fertilities (the response variable) are not directly measured but estimated (Smouse et al. 1999; Morgan and Conner 2001). The spatially explicit mating model (Oddou-Muratorio et al. 2005; Austerlitz et al. 2012) allows estimating male selection gradients directly. The strength of this method consists in computing jointly direct selection gradients for the male function, together with the selfing rate, the pollen immigration rate (thus controlling for the effect of pollen flow from unsampled individuals) and a pollen dispersal function for outcrossing events (dispersal kernel) representing

the dependency of pollen flow upon the distance from the source plant (Oddou-Muratorio et al. 2005; Gérard et al. 2006; Fénart et al. 2007; Austerlitz et al. 2012).

In this study, we estimated direct selection gradients for the male function in a wild population of the white-flowered *Petunia axillaris* (Lam.) Britton, Sterns and Poggenb. *Petunia* (Solanaceae) is a long-standing research model (Sink 1984; Gerats and Vandenbussche 2005). However, despite being extensively studied, most research on reproductive biology in this taxon has been carried out using hybrids, and studies on natural populations are scarce (but see Dell'Olivo et al. 2011). Of particular relevance, Brandenburg et al. (2012) found that variation in nectar volume of *P. axillaris* affects female reproductive success, measured as seed set. Nevertheless, this study considered extreme nectar variation as it compared a near-isogenic line with low nectar production with a few wild plants, and it did not examine selection via male function. Here we studied a natural population of *P. axillaris* and specifically asked (1) whether natural variation in nectar-related traits also affects male reproductive success under natural pollination, and (2) whether selection can occur on nectar indirectly, through phenotypic correlations between nectar and traits related to pollinator attraction, access to rewards, and reproductive investment that may also be subject to phenotypic selection through male function. To address these questions, we used a spatially explicit analysis (Oddou-Muratorio et al. 2005; Austerlitz et al. 2012) that described pollen dispersal patterns and estimated phenotypic selection via male fitness directly.

Materials and methods

Study species and site

Petunia axillaris axillaris (*P. axillaris* hereafter) is a white-flowered petunia that comprises self-compatible and self-incompatible populations (Tsukamoto et al. 2003; Kokubun et al. 2006). Although the species is perennial, it usually behaves as an annual plant under natural conditions (Dell'Olivo et al. 2011). Flowers produce nectar containing glucose, fructose and sucrose (but no amino acids; Brandenburg et al. 2012), which is secreted continuously throughout a flower lifetime (Galleto and Bernardello 1993). In the absence of pollination, flowers last for several days (Oyama-Okubo et al. 2005). As flowers open and become strongly fragrant at night, the species was first considered to be nocturnally pollinated (Ando et al. 2001; Hoballah et al. 2005); however, diurnal pollination has since been shown to be equally effective (Hoballah et al. 2007; Dell'Olivo et al. 2011). Flower visitors observed in natural populations in Uruguay include moths belonging to the genera *Manduca*, *Eumorpha*, *Agrius* and *Erinnys*, bees from the genera *Halictus* and *Lasioglossum*, and beetles from the genera *Diabrotica*, *Chrysodina* and *Dahlibruchus* (Hoballah et al. 2007).

We sampled a population growing on sandy dunes along the shore of Sauce de Portezuelo, Uruguay (34°52′41.8″S, 55°08′28.4″W; Fig S1), in November 2010, during peak flowering. The population contained 311 plants, and occupied a 70 × 80 m plot with no other individuals within 400–500 m radius (Electronic Supplementary Material Fig S2a,b). We recorded individual plant positions in a two-dimensional coordinate system (Electronic Supplementary Material Fig. S3). We collected leaves for DNA extraction from all plants in the plot, and mature unopened fruits from all plants that presented fruits in this developmental stage at the time of the population survey. From these fruits we collected the seeds for paternity analyses (see below). All phenotypic measurements were performed

after fruit collection so that the sampled seeds resulted from undisturbed pollination patterns.

Phenotypic measures

We measured phenotypic traits in 92 individuals presenting both new flowers and developing fruits (Electronic Supplementary Material Fig S2a). In addition to floral rewards (nectar volume and concentration), we also measured for each plant its size, as well as traits related to pollinator attraction (floral display and corolla area), access to nectar (corolla tube length), and direct investment in male function (number and average size of pollen grains per anther).

We measured floral traits in a total of 576 newly opened flowers, with an average (±SD) of 6 (±3) flowers per plant. Flowers open at dusk, so we controlled for flower age by bagging flower buds with fine mesh bags and collecting all newly and fully opened bagged flowers every evening between 8 and 10 pm. Bagging prevented pollinator access to either nectar or pollen, thus ensuring accurate estimates of nectar volume and concentration as well as number of pollen grains per anther (see below).

Nectar volume per flower was measured by centrifugation (Stuurman et al. 2004). We cut the corolla tube where the stamens detached from the tube, and placed it in a 0.5 ml Eppendorf tube. The Eppendorf tube was pierced with three holes and centrifuged inside a larger, 1.5 ml Eppendorf tube (3,500 rpm for 5 min). Nectar was extracted and, because of centrifugation, it collected itself in the outer tube, where its volume could be measured after removing the inner tube containing the empty flower. Nectar volume was measured with a calibrated pipette. *Nectar concentration* was measured with a Fisher Scientific hand-held refractometer in the range of 0–32 % Brix (±0.2 %: mg of solute/mg solution accuracy); nectar was diluted 1:1 before being measured. We corrected each concentration measurement for the ambient air temperature (min/max = 16/33 °C).

To obtain standardized measurements of *corolla area*, we photographed the corolla of each flower in a dark photobox which maintained a constant distance to the camera (Electronic Supplementary Material Fig S4). *Corolla area* was measured from these photographs using the ImageJ software (Abramoff et al. 2004). We measured *corolla tube length* using a ruler (±0.1 cm accuracy) as the distance from the base of the gynoecium to the point at which the corolla began flaring (Stuurman et al. 2004; Electronic Supplementary Material Fig S5).

The *number of pollen grains per anther* was counted for indehiscent anthers which had been stored in Eppendorf tubes in 70 % ethanol. Each tube was sonicated for 1 min and the anther remains were removed with ethanol. The contents of each Eppendorf tube were then transferred into a 30 ml glass counting chamber filled with 0.63 % NaCl solution. The pollen grains were counted with an electronic particle counter (Particle Data Elzone 180XY; Micromeritics), which counted the number of particles with a diameter between 12 and 77 μm in three 1 ml subsamples, and assigned them to 128 logarithmic diameter classes.

We estimated *plant size* by counting the total number of branches on each plant, and an integrative measure of *floral display size,* as the sum of open flowers, wilted flowers and flowering scars at the time of fruit collection. The sampled plants had consistent floral display size over time, as the number of open flowers at the time of fruit collection was positively correlated to both the number of wilted flowers (Spearman $r = 0.45$, $N = 92$, $P < 0.001$) and the number of flowering scars (Spearman $r = 0.48$, $N = 92$, $P < 0.001$).

Thus, our estimation seemed representative of the variation in floral display size among plants at the time when pollinator visitation led to the formation of the genotyped seeds.

Finally, to assess whether siring success trades-off with an investment in the female function, we counted the *number of seeds per fruit* ($N = 39$ fruits from different plants) by using a seed counter (Elmor C3, Elmor Applied Electronics, Switzerland), and included this trait as a covariate in the male selection gradient analysis (see below). Phenotypic correlations between all the measured traits were estimated as partial Spearman's correlation coefficients using the R package *ppcor* (Kim and Yi 2007; R Core Team 2014).

Genotyping

Genotypes were obtained using six microsatellite loci; the primer sequences were obtained from Bossolini et al. (2011). Genomic DNA was extracted from dried leaves using the Qiagen DNeasy Plant Mini Kit. PCRs were conducted in a 10 µl mixture containing approximately 5–10 ng of template DNA, 2 µM of forward and reverse primers, 0.5 µg/µl BSA and 1× Qiagen HotStarTaq *Plus* Master Mix. Amplifications were performed in a Biometra thermal cycler and involved an initial activation step of 15 min at 95 °C, followed by *n* cycles comprising 30 s of denaturation at 94 °C, 90 s of annealing at *T*, 60 s of extension at 72 °C, and a final extension step of 30 min at 60 °C (see Table 1 for the *n* and *T* values for each locus). The amplified products were separated on an ABI PRISM 3100 genetic analyzer (Applied Biosystems), and sizes were assigned with the GENEMAPPER v3.7 (Applied Biosystems) software, using Genescan-350 as the internal size standard. Allele binning was performed with the TANDEM software (Matschiner and Salzburger 2009).

We genotyped all 311 adult plants present in the population (Table 1 provides a description of the microsatellite loci used and their exclusion power) and assessed their paternal contributions to seeds produced by 30 individuals (*maternal plants* hereafter) from the dense part of the population (Electronic Supplementary Material Fig S2b). We focused on these maternal plants to limit the proportion of pollen flow from unsampled individuals, increasing the resolution of our analysis. We estimated paternity on 20 (SD ± 2) seeds germinated from one fruit per maternal plant (604 seedlings in total); the offspring genotypes were obtained using the same protocols for DNA extraction and PCR as for the adult plants. By applying the PATRI software (Nielsen et al. 2001; Signorovitch and Nielsen 2002) on the genetic data, we estimated that there were a total of 523 breeding males. According to this estimate, the sampled individuals would represent 60 % of the true breeding population (i.e. all the plants siring the sampled offspring).

Paternity analyses

We estimated selection gradients based on siring success with a spatially explicit mating model (SEMM hereafter; Oddou-Muratorio et al. 2005, 2006; Austerlitz et al. 2012) which stems from the neighbourhood model (Adams and Birkes 1991; Burczyk et al. 2002, 2006). This approach estimates the effects of measured covariates on the male fecundity of individuals, jointly with the selfing (*s*) and pollen immigration (*m*) rates, while also accounting for the spatial position of the sampled plants. The method, which is related to the fractional attribution of paternity (Jones et al. 2010), models paternity as relative paternal contributions to maternal pollen clouds (see Oddou-Muratorio et al. 2005). It differs from the classical selection gradient approach in which individual fitness is measured or estimated, as fitness is modelled with a likelihood function which is optimized in

Table 1 Description of the six *Petunia axillaris* microsatellite loci used in the paternity analyses

Locus[a]	Repeat motif[a]	T (°C)[a]	n[a]	Ar[a]	R_s[b]	R_m[b]	R_o[b]	H_O[a]	H_E[a]	HW[c]	F_{null}[d]	EP[d]	*GenBank*[a]
PM17	$(CTG)_8$	54	38	176–188	3.228	3.000	2.993	0.492	0.497		0.009	0.258	CV301045
PM107	$(CAA)_8$	50	40	119–134	4.036	3.929	3.590	0.553	0.565		0.012	0.322	FN001301
PM119	$(TG)_9$	54	40	149–163	2.776	2.984	2.861	**0.345**	**0.381**	*	0.048	0.169	FN004737
PM149[e]	$(CT)_{10}$	50	38	101–117	7.395	6.000	6.749	**0.497**	**0.762**	**	0.209	0.560	FN042637
PM188	$(CTT)_8$	50	38	102–126	7.287	6.865	6.940	**0.872**	**0.829**	**	−0.028	0.653	FN037917
PM206	$(TC)_{12}$	54	38	104–124	6.287	6.000	6.000	0.813	0.797		−0.010	0.597	FN035807

Significant differences between *Ho* and *He* are in bold with * $P < 0.05$; ** $P < 0.005$

[a] Locus name, repeat motif, annealing temperature (T), number of cycles in the PCR (n), allele size range (Ar), observed (H_O) and expected (H_E) heterozygosity, and GenBank accession number

[b] Per locus allelic richness for all potential sires (R_s), maternal plants (R_m) and offspring (R_o) were estimated with FSTAT v 2.9.3.2 (Goudet 1995)

[c] Departures from Hardy–Weinberg equilibrium (*HW*) were analyzed following the algorithm described in Guo and Thompson (1992), as implemented in ARLEQUIN v3.1 (Excoffier et al. 2005), using 100,000 steps in the Markov chain and 1,000 dememorisation steps

[d] Null allele frequencies (F_{null}) and exclusion probabilities when one parent (mother) is known (*EP*) were estimated with CERVUS v 3.0 (Marshall et al. 1998; Kalinowski et al. 2007); the combined exclusion probability across all loci, given a known maternal genotype, was 0.968

[e] Al homozygote genotypes for this locus were recoded as heterozygotes possessing the detected allele and a null one, following Jones and Ardren (2003), to avoid incorrect paternity inference due to null alleles

order to estimate population level parameters (see Electronic Supplementary Material), allowing direct estimation of paternal selection gradients (e.g. Morgan and Conner 2001; van Kleunen and Burczyk 2008).

The spatial component of the applied SEMM was modelled with an Exponential Power dispersal kernel characterizing the probability of pollen dispersal between two plants as a function of their spatial proximity (the Geometric, Weibull and 2Dt kernels resulted in lower likelihoods; data not shown), as

$$p_{ep}(a,b;x,y) = \frac{b}{2\pi a^2 \Gamma(2/b)} \exp\left(-\left(\frac{r}{a}\right)^b\right), \quad (1)$$

where Γ is the complete gamma function and r the distance between two plants (Austerlitz et al. 2004). This kernel is characterized by a, the scale parameter, which determines the average dispersal distance (δ), and b, the shape parameter, which describes the tail of the curve ($b < 1$ indicates that long-distance dispersal can occur; Austerlitz et al. 2004). These dispersal parameters were estimated jointly with the immigration (m) and selfing (s) rates, and with the regression coefficients for the phenotypic covariates. The effect of trait i was modelled by considering both linear (β_i: directional) and quadratic (γ_i: stabilizing or disruptive) selection gradients (see e.g. Wright and Meagher 2004). Thus, the phenotypic selection gradient associated with trait i for male k was

$$\ln(f_i(z_{ki})) = \beta_i z_{ki} + \gamma_i z_{ki}^2, \quad (2)$$

where $f_i(z_{ki})$ is the multiplicative male fecundity component. If γ_i was not significant, a positive (resp. negative) value for β_i meant that an increased value of the trait had always a positive (resp. negative) effect on male fecundity. If γ_i was significant and negative, the trait was under stabilizing selection (male fecundity was maximal for an intermediate value of the trait), while if γ_i was significant and positive, it indicated disruptive selection (male fecundity was maximal for the lowest and highest value of the trait and minimal for an intermediate value).

All parameters (a, b, m, s, and all β_i and γ_i values) were estimated jointly by computing a single likelihood function (see Electronic Supplementary Material) that combined Eqs. (1) and (2). This likelihood function was maximised using a numerical method (allowing for a maximum of 100,000 iterations) implemented in a MATHEMATICA (Wolfram Research) notebook (available from FA). We assumed a genotyping error rate of 1 % for all loci, and that genotyping errors yielded an allele of any size (as in Llaurens et al. 2008).

Phenotypic values (z_{ki}) were standardized to zero mean and unit variance, to allow comparison of the effects of the covariates. Plants that were genotyped as putative sires, but that had not been measured for trait i, were represented by $z_{ki} = 0$, which corresponds to the population mean. As a result, the male fecundity components $f_i(z_{ki})$ of the individuals with missing values for trait i were equal to one, whatever the values of β_i and γ_i. Hence, these individuals did not contribute to the likelihood function, and estimation of β_i and γ_i depended only on individuals with measured values of trait i.

The significance of each parameter estimate was evaluated with Likelihood Ratio Tests (LRTs; see Oddou-Muratorio et al. 2005 for more details). As they corresponded to a type III analysis where the variables were removed one by one, these tests evaluated the significance of each factor independently, even if some of these factors were partially correlated. We also assessed the existence of overall selection on each trait with LRTs that compared the complete model against reduced models in which both the linear and the quadratic terms for a trait were removed simultaneously.

To illustrate the heterogeneity in male fecundity, we also estimated the relative individual male fecundity of each plant using a Bayesian mating model implemented in the software MEMM v 1.1 (MEMM hereafter; Klein et al. 2008, 2011). This approach is similar to the SEMM described above, as it models the spatial dependency of siring success through pollen dispersal kernels, but it considers the individual fecundities as a random effect, and estimates them using a Bayesian approach that assumes that all fecundities are drawn from a log normal distribution with unit mean and variance σ^2; this variance being jointly estimated with the individual fecundities (see Klein et al. 2008 for more details).

Results

Phenotypic variation and correlations

Nectar characteristics, as well as the other measured traits, varied extensively among the 92 phenotyped plants, and among flowers within plants (Electronic Supplementary Material Table S1). In particular, plant size (2–309 branches) and floral display size (8–2,063 flowers) varied the most, owing to a few very large individuals. Variation in floral traits ($N = 576$ flowers) among plants exceeded variation within plants, as evidenced by the significant repeatability for all floral trait measurements (Electronic Supplementary Material Table S2). Phenotypic correlation analysis revealed that larger plants produced more flowers (*partial Spearman* $r = 0.85$, $P \ll 0.01$; Table 2). Given this strong correlation, we avoided collinearity by including floral display size but not plant size as a covariate in the selection gradient analyses (see below). Floral display size did not correlate significantly with either measure of floral size (corolla area and corolla tube length; Table 2, Electronic Supplementary Material Fig. S4, S5), and so it was not apparently involved in a trade-off, although plant resource status was not controlled for. Most importantly, we could not detect any correlation between nectar traits and other floral traits (Table 2).

Mating patterns and selection analyses

The parameters estimated for the exponential power kernel indicated that mean pollination distance was limited ($\delta = 12.40$ m), and that the probability of pollination decreased more slowly with distance than expected from an exponential decline ($b = 0.32$). Thus, most pollen dispersed locally, but long-distance dispersal within the study population also occurred at a non-negligible rate. Pollen immigration into the sampled population was low ($m = 0.19$). The selfing rate was estimated at zero, in agreement with previous evidence that all southernmost populations of *P. axillaris* in Uruguay are completely self-incompatible (Kokubun et al. 2006).

Individual male fecundities, as estimated with MEMM (Klein et al. 2011), were highly heterogeneous among adult plants, with most individuals having low fecundities and only a few individuals being very fecund (Fig. 1). Differences in male fecundities could be significantly explained by phenotypic differences among individuals (Table 3, Electronic Supplementary Material Fig. S6). Of the two measured nectar traits, nectar volume showed no significant selection ($P = 0.61$), whereas stabilizing selection was evident for nectar concentration ($\beta = 2.23$, $P < 0.01$ and $\gamma = -0.76$, $P = 0.02$) with maximum male fecundity reached for a nectar concentration of 44.6 % Brix. Stabilizing selection was also found for floral display size ($\beta = 2.20$, $P < 0.01$ and $\gamma = -0.22$, $P = 0.01$), reaching a

Table 2 Pairwise partial correlation coefficients for reproductive traits measured in *Petunia axillaris*

	Floral display size	Corolla area	Corolla tube length	Nectar volume	Nectar concentration	Pollen grains/ anther	Pollen size	Seeds/ fruit
Plant size	**0.846**	−0.107	0.233	−0.225	−0.024	0.114	0.007	−0.028
Floral display size		<0.001	−0.089	0.149	0.072	−0.108	−0.085	0.054
Corolla area			**0.343**	0.277	−0.075	0.072	−0.093	0.067
Corolla tube length				0.264	−0.135	−0.009	0.118	0.044
Nectar volume					0.143	0.012	0.139	0.096
Nectar concentration						−0.008	0.059	−0.025
Pollen grains/ anther							0.009	0.046
Pollen size								−0.005

Significant correlations, after sequential Bonferroni correction applied to account for multiple pairwise comparisons (Holm 1979), are indicated in bold

maximum at about 1,600 flowers. Corolla area and corolla tube length were both subjected to directional selection, with male fecundity increasing with corolla area ($\beta = 1.14$, $P < 0.01$) and decreasing with corolla tube length ($\beta = -0.75$, $P = 0.03$). Interestingly, male fecundity also exhibited a quadratic relation to the number of seeds per fruit ($\beta = 1.76$, $P < 0.01$ and $\gamma = -0.80$, $P = 0.03$), with a peak at 800 seeds per fruit.

Discussion

Despite the incontestable role that nectar traits play in zoophilous pollination, few studies have analysed the effects of nectar variation on male fitness (e.g. Mitchell 1993; Hodges 1995; Johnson et al. 2004; Kulbaba and Worley 2012), and hardly ever by estimating realized male reproductive success in an ecologically relevant, natural context. We analyzed here the effect of natural variation in nectar traits on male fecundity in a wild *P. axillaris* population by applying a paternity-based direct selection gradient analysis, and found that nectar concentration is under stabilizing selection through male function. We also considered the effects of other key reproductive traits on male fitness, and showed that the effect of nectar occurred independently of the effect of any other studied floral trait. More generally, variation in floral traits accounted for at least a portion of the spatial variation in male fitness we reported here.

Gene flow and mating patterns in *Petunia axillaris*

The spatially explicit analysis of paternity applied to trace pollen movements within the study population detected around 20 % of pollen immigration, showing that the sampling effort and the power of the molecular markers allowed the detection of most successful pollination events. We also inferred a selfing rate of zero. The absence of selfing, despite phenological opportunities for self-pollination (GG and AI; personal observation), suggests the existence of a genetic mechanism of self-incompatibility, as described for other *P.*

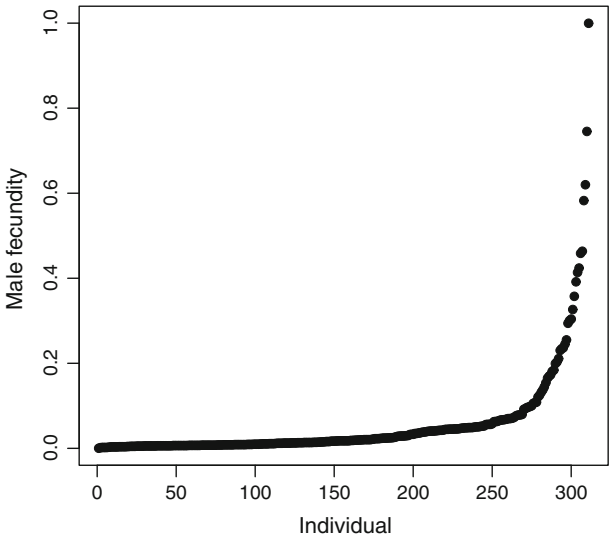

Fig. 1 Relative individual male fecundities ordered by increasing values ($N = 311$ plants). Male fecundity values were standardized with respect to the highest value estimated

axillaris populations (Tsukamoto et al. 2003). The estimated mean pollen dispersal distance was 12 m, indicating that successful pollination mainly occurred among neighbouring plants. It should be noted, however, that as mother plants were sampled in the dense part of the patch, the pollen dispersal patterns we observed result probably to some extent from our sampling design. Nevertheless, rare long-distance pollination events also occurred, as shown by the incidence of pollen immigration and the fat-tailed exponential power kernel. *P. axillaris* grows in unstable environments forming ephemeral patches. In this context, even if most pollination occurs within a patch, long-distance dispersal may be crucial to counter genetic erosion and favour local population persistence, as it provides genetic connections among patches.

Selection on nectar traits

Male fecundity varied extensively (Fig. 1). Uneven contributions to paternity are frequently observed in plants (Elle and Meagher 2000; Wright and Meagher 2004; Hodgins and Barrett 2008; Klein et al. 2008; Iwaizumi et al. 2013) and result in fewer-than-expected effective fathers (i.e. those effectively contributing to gene pool). As shown by the SEMM analysis, this heterogeneity in reproductive success was significantly explained by phenotypic differences among individuals in nectar concentration, and other traits like floral display, corolla area and tube length, which did not covary with nectar concentration. Interestingly, we did not find an effect of nectar volume on male fecundity.

The SEMM analysis revealed, in particular, a stabilizing selection gradient for nectar concentration. Nectar is probably the main plant reward for pollinators, and its concentration determines both the energetic return per volume unit, and, through its effects on viscosity, the energy and time costs to extract a given nectar volume (Heyneman 1983; Harder 1986). Since nectar-feeding animals from different taxonomic groups prefer different nectar concentrations associated with different behavioural and physiological

Table 3 Linear (β) and quadratic (γ) coefficients of the male selection gradient analysis estimated by applying a spatially explicit mating model of paternity (SEMM)

Covariate[a]	Estimated gradients[a]		Significance testing (reduced model lacking either β or γ)[b]		Significance testing (reduced model lacking both β and γ)[c]
	Linear gradient (β)	Quadratic gradient (γ)[d]	P ($\beta \neq 0$)	P ($\gamma \neq 0$)	P
Corolla area	**1.142**	−0.415	**0.002**	0.137	**0.002**
Corolla tube length	**−0.75**	−0.013	**0.032**	0.94	**0.045**
Nectar volume	0.246	−0.132	0.269	0.649	0.614
Nectar concentration	**2.23**	**−0.764**	**<0.001**	**0.018**	**<0.001**
Number of pollen grains/anther	−0.044	0.223	0.815	0.067	0.185
Pollen size	−0.299	0.308	0.166	0.146	0.133
Floral display size	**2.197**	**−0.217**	**<0.001**	**0.014**	**<0.001**
Seeds/fruit	**1.757**	**−0.798**	**<0.001**	**0.033**	**<0.001**

Significant values ($P < 0.05$) are highlighted in bold

[a] Phenotypic data were standardized to a zero mean and unit variance

[b] The significances of the β and γ coefficients were evaluated independently by Likelihood Ratio tests, comparing the complete model (with all parameters included) with a reduced model in which each parameter was removed in turn

[c] The effect of each covariate was tested globally by performing Likelihood Ratio tests that compared the complete model with a reduced model in which both the β and γ terms were removed simultaneously

[d] Values were estimated following Eq. (2), and thus represent unadjusted quadratic regression coefficients as described in Stinchcombe et al. (2008)

capabilities (Kevan and Baker 1983; Kim et al. 2011), nectar concentration is expected to be under selection driven by the feeding preferences of the most effective pollinators. *P. axillaris* is pollinated both by nocturnal and diurnal pollinators (Hoballah et al. 2007; see also "Materials and methods"). In the study population, the mean concentration was around 35 %, close to the optimal nectar concentration reported for hawkmoths and some bees (around 30–40 %; Kim et al. 2011). However, according to our analysis, male fecundity was highest for a nectar concentration around 44 %, suggesting that other pollinators consuming more concentrated nectar could also successfully pollinate *P. axillaris*. In addition, optimum nectar concentration could reflect a balance between pollinator-mediated selection and the optimum value to escape damage by antagonists that are also attracted to nectar (Maloof and Inouye 2000; Galen and Butchart 2003; Adler and Bronstein 2004). None of the flowers examined was damaged, indicating an absence or a very low incidence of nectar robbery; however, we cannot discard an antagonistic effect of nectar thieves, which do not damage flowers (Inouye 1980).

Surprisingly, we did not find any evidence that nectar volume is under selection through male fitness, as it has been reported in other studies (e.g. Mitchell 1993; Hodges 1995). In *P. axillaris*, nectar is concealed at the base of the corolla tube. Given the absence of significant correlations among nectar traits and floral display or corolla morphology, there seems to be no external cue reflecting either nectar quantity or quality. Thus, we assume that pollinators likely have to probe flowers to assess nectar properties; the amount of pollen removed or deposited on stigmas may be a function of the time pollinators spend

feeding on nectar. Indeed, in a recent laboratory experiment, *Manduca sexta* hawkmoths (one of *P. axillaris* main pollinators) spent less time per flower on *P. axillaris* plants containing an introgressed low-nectar volume locus than on wild plants, resulting in lower seed set in the low-nectar volume plants than in normally secreting wild plants (Brandenburg et al. 2012). In contrast, nectar volume variation in the study population did not affect male fecundity to any significant extent. This may be a consequence of the fact that extremes in nectar secretion could have already been curtailed by selection, as too low nectar production would limit pollen removal whereas too high nectar production would increase self and geitonogamous pollination (e.g. Hodges 1995), and thus reduce the fraction of exported pollen (i.e. pollen discount). Still, nectar volume appears to be relatively variable compared to most other characters (Table S1); maintaining such variability could be adaptive as unreliable rewards among plants may promote outcrossing (Paccini and Nepi 2007). These two hypothesis are not-mutually exclusive and either one would predict a mostly flat selection gradient.

Selection on other phenotypic traits and functional trade-offs

Independently of nectar effects, we found significant selection gradients for corolla area and floral display, indicating, as in other studies (e.g. Morgan and Conner 2001; Austerlitz et al. 2012), that increased allocation to attractive structures enhances the male function. The corolla limb area was under positive directional phenotypic selection, consistent with behavioural experiments with introgressed petunias differing just in corolla area, which found that hawkmoth pollinators prefer large over small corollas (Venail et al. 2010). On the other hand, floral display size was under stabilizing selection. However, the maximum value in the parabolic curve exceeded largely the observed population mean, suggesting a tendency for selection for larger floral displays, consistent with a general preference of pollinators (Ohashi and Yahara 2001), and probably reflecting resource limitations, commonly observed in many naturally growing plants. The reduced male fecundity of plants with extreme floral display sizes may reflect either a trade-off between flower production and allocation to attractive traits not considered here, pollen discounting effects (as geitonogamy usually increases with floral display size; Harder and Barrett 1995; Karron and Mitchell 2012), or the fact that pollinator visits usually increase in a decelerating manner with increasing floral display size (Ohashi and Yahara 1998).

Only corolla tube length experienced negative directional selection. Such selection is consistent with a behavioural experiment with the pollinator *M. sexta* performed in laboratory, in which the hawkmoths displayed an innate preference for shorter corolla tubes (Venail et al. 2010). These hawkmoths have proboscides that largely exceed corolla depths of *P. axillaris*, so they do not face a morphological impediment to access nectar. However, in the laboratory, the hawkmoths chose mainly short-tubed flowers, and they made their choice before extending their proboscis, so they seemed capable of screening tube lengths before probing the flowers (Venail et al. 2010). Therefore, the higher pollen export in short-tubed plants in the study population could probably reflect higher visitation rates by hawkmoths.

Interestingly, the SEMM analysis detected a quadratic relationship between male fecundity and seeds per fruit. This pattern reflected a trade-off between male and female functions, which became evident only in plants that invested heavily into female function. Trade-offs between the two sexual functions have been already found (e.g. Mazer et al. 1999), but to our knowledge, this is the first study finding such trade-offs by estimating directly the effect that allocation to female function has on male fecundity.

Selection gradients and evolutionary directions

Evolutionary change requires heritability of phenotypic variation and also that the traits under selection are not constrained by genetic correlations with other traits under selection. In *Petunia,* QTL mapping has begun to unveil the genetic architecture of quantitative floral traits (Stuurman et al. 2004; Galliot et al. 2006; Venail et al. 2010). In particular, loci controlling corolla size and tube length are not tightly linked (Venail et al. 2010); thus, it is unlikely that a genetic correlation between these two traits could explain the divergent selection we detected for these two traits. On the other hand, QTL mapping using *P. integrifolia* and *P. parodii* as the pure parental petunias detected a genetic correlation between corolla size and nectar volume (Stuurman et al. 2004). Therefore, in *P. axillaris*, indirect selection on nectar volume may occur via positive selection on corolla area. However, as QTL studies involved interspecific crosses, they may not necessarily describe genetic correlations and heritability in natural populations (Conner 2002). Given the high potential for phenotypic selection detected here, estimation of heritability and genetic correlations in wild *P. axillaris* populations is warranted.

In conclusion, our results show that natural variation in nectar concentration, as well as in other key reproductive traits, affects male fitness in *P. axillaris*. An important limitation of our study is the absence of data on flower visitors and on their behaviour, which limits interpretation of the possible mechanisms that may generate the observed patterns. In addition, some of our estimated selection gradient coefficients were larger than those usually obtained applying classical approaches (e.g. Harder and Johnson 2009). This discrepancy likely stems from the fact that fitness was not measured as in classical selection gradient studies, but was modelled through a mating model; indeed, van Kleunen and Burczyk (2008) obtained male selection gradients estimates of similar magnitude as ours using a comparable approach. Furthermore, although correlation among traits is controlled for in the multiple regression approaches we applied, we cannot exclude that additional correlated traits not included in our study may also be relevant for male fitness (Conner and Hartl 2004). Introgression lines that differ only in specific traits are thus one of the most promising tools (Bradshaw and Schemske 2003; Brandenburg et al. 2012), particularly in combination with exposure to natural pollinators, observation of flower visitors and parentage-based fitness assessment.

Acknowledgments We thank Marcelo Aizen, Redouan Bshary, Lawrence Harder, Cris Kuhlemeier, three anonymous reviewers and the editors for comments, Dessislava Savova-Bianchi, Eligio Bossolini, José Gabriel Segarra-Moragues, Leonardo Galetto, Portia Lloyd, Mariana Musicante and Denis Pernecker for practical help, and Lawrence Harder for access to the particle counter. This study was supported by the Swiss NSF (Grant No. 3100A0_122004/1 to GB, Grant No. IZK0Z3-125693 to FA and GB) and the Fondation Mercier pour la Science. GG is a member of the Consejo Nacional de Investigaciones Científicas y Técnicas from Argentina. This project benefitted intellectually from collaboration with Cris Kuhlemeier and Redouan Bshary within the framework of the National Centre of Competence in Research (NCCR) Plant Survival (Work package 2.2), a program of the Swiss NSF.

References

Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int 11:36–42

Adams WT, Birkes DS (1991) Estimating mating patterns in forest tree populations. In: Fineschi S, Mavolti ME, Cannata F, Hattemer HH (eds) Biochemical markers in the population genetics of forest trees. Academic Publishing, The Hague, pp 157–172

Adler LS, Bronstein JL (2004) Attracting antagonists: Does floral nectar increase leaf herbivory? Ecology 85:1519–1526
Ando T, Nomura M, Tsukahara J, Watanabe H, Kokubun H, Tsukamoto T, Hashimoto G, Marchesi E, Kitching IJ (2001) Reproductive isolation in a native population of *Petunia* sensu Jussieu (Solanaceae). Ann Bot 88:403–413
Austerlitz F, Dick CW, Dutech C, Klein EK, Oddou-Muratorio S, Smouse PE, Sork VL (2004) Using genetic markers to estimate the pollen dispersal curve. Mol Ecol 13:937–954
Austerlitz F, Gleiser G, Teixeira S, Bernasconi G (2012) The effects of inbreeding, genetic dissimilarity and phenotype on male reproductive success in a dioecious plant. Proc R Soc B Biol Sci 279:91–100
Bell G (1985) On the function of flowers. P Roy Soc B Biol Sci 224:223–265
Bernasconi G (2003) Seed paternity in flowering plants: an evolutionary perspective. Perspect Plant Ecol Evol Syst 6:149–158
Bossolini E, Klahre U, Brandenburg A, Reinhardt D, Kuhlemeier C (2011) High resolution linkage maps of the model organism *Petunia* reveal substantial synteny decay with the related genome of tomato. Genome 54:327–340
Bradshaw HD, Schemske DW (2003) Allele substitution at a flower colour locus produces a pollinator shift in monkey flowers. Nature 426:176–178
Brandenburg A, Kuhlemeier C, Bshary R (2012) Hawkmoth pollinators decrease seed set of a low-nectar *Petunia axillaris* line through reduced probing time. Curr Biol 22:1635–1639
Burczyk J, Adams WT, Moran GF, Griffin AR (2002) Complex patterns of mating revealed in a *Eucalyptus regnans* seed orchard using allozyme markers and the neighbourhood model. Mol Ecol 11:2379–2391
Burczyk J, Adams WT, Birkes DS, Chybicki IJ (2006) Using genetic markers to directly estimate gene flow and reproductive success parameters in plants on the basis of naturally regenerated seedlings. Genetics 173:363–372
Campbell DR (1989) Measurements of selection in a hermaphroditic plant: variation in male and female pollination success. Evolution 43:318–334
Carlson JE (2007) Male-biased nectar production in a protandrous herb matches predictions of sexual selection theory in plants. Am J Bot 94:674–682
Conner JK (2002) Genetic mechanisms of floral trait correlations in a natural population. Nature 420:407–410
Conner JK (2006) Ecological genetics of floral evolution. In: Harder LD, Barrett SCH (eds) The ecology and evolution of flowers. Oxford University Press, Oxford, pp 260–277
Conner JK, Hartl DL (2004) A primer of ecological genetics. Sinauer Associates, Sunderland
Conner JK, Rush S, Kercher S, Jennetten P (1996) Measurements of natural selection on floral traits in wild radish (*Raphanus raphanistrum*). II. Selection through lifetime male and total fitness. Evolution 50:1137–1146
Cuartas-Domínguez M, Medel R (2010) Pollinator-mediated selection and experimental manipulation of the flower phenotype in *Chloraea bletioides*. Funct Ecol 24:1219–1227
Dell'Olivo A, Hoballah ME, Gübitz T, Kuhlemeier C (2011) Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). Evolution 65:1979–1991
Elle E, Meagher TR (2000) Sex allocation and reproductive success in the andromonoecious perennial *Solanum carolinense* (Solanaceae). II. Paternity and functional gender. Am Nat 156:622–636
Endler J (1986) Natural selection in the wild. Princeton University Press, New Jersey
Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50
Fénart S, Austerlitz F, Cuguen J, Arnaud J-F (2007) Long distance pollen-mediated gene flow at a landscape level: the weed beet as a case study. Mol Ecol 16:3801–3813
Galen C, Butchart B (2003) Ants in your plants: effects of nectar-thieves on pollen fertility and seed siring capacity in the alpine wildflower, *Polemonium viscosum*. Oikos 101:521–528
Galleto L, Bernardello L (1993) Nectar secretion pattern and removal effects in three species of Solanaceae. Can J Bot 71:1394–1398
Galliot C, Stuurman J, Kuhlemeier C (2006) The genetic dissection of floral pollination syndromes. Curr Opin Plant Biol 9:78–82
Gérard PR, Klein EK, Austerlitz F, Fernandez-Manjarres JF, Frascaria-Lacoste N (2006) Assortative mating and differential male mating success in an ash hybrid zone population. BMC Evol Biol 6:96
Gerats T, Vandenbussche M (2005) A model system for comparative research: *Petunia*. Trends Plant Sci 10:251–256
Goudet J (1995) FSTAT (vers. 1.2): A computer program to calculate F-statistics. J Hered 86:485–486
Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48:361–372

Harder LD (1986) Effects of nectar concentration and flower depth on flower handling efficiency of bumble bees. Oecologia 69:309–315
Harder LD, Barrett SCH (1995) Mating cost of large floral displays in hermaphrodite plants. Nature 373:512–515
Harder LD, Johnson SD (2009) Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytol 183:530–545
Harder LD, Routley MB (2006) Pollen and ovule fates and reproductive performance by flowering plants. In: Harder LD, Barrett SCH (eds) The ecology and evolution of flowers. Oxford University Press, Oxford, pp 61–80
Heyneman AJ (1983) Optimal sugar concentrations of floral nectars. Dependence on sugar intake efficiency and foraging costs. Oecologia 60:198–213
Hoballah ME, Stuurman J, Turlings TC, Guerin PM, Connetable S, Kuhlemeier C (2005) The composition and timing of flower odour emission by wild *Petunia axillaris* coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*. Planta 222:141–150
Hoballah ME, Gubitz T, Stuurman J, Broger L, Barone M, Mandel T, Dell'Olivo A, Arnold M, Kuhlemeier C (2007) Single gene-mediated shift in pollinator attraction in *Petunia*. Plant Cell 19:779–790
Hodges SA (1995) The influence of nectar production on hawkmoth behaviour, self pollination, and seed production in *Mirabilis multiflora* (Nyctaginaceae). Am J Bot 82:197–204
Hodgins KA, Barrett SCH (2008) Natural selection on floral traits through male and female function in wild populations of the heterostylous daffodil *Narcissus triandrus*. Evolution 62:1751–1763
Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6:65–70
Inouye DW (1980) The terminology of floral larceny. Ecology 61:1251–1253
Iwaizumi MG, Takahashi M, Isoda K, Austerlitz F (2013) Consecutive multiple-year analysis of paternal and maternal gene flow and contributions of the gametic heterogeneities to overall genetic composition of *Pinus densiflora* dispersed seeds. Am J Bot 100:1896–1904
Johnson SD, Peter CI, Ågren J (2004) The effects of nectar addition on pollen removal and geitonogamy in the non-rewarding orchid *Anacamptis morio*. Proc R Soc B Biol Sci 271:803–809
Jones AG, Ardren WR (2003) Methods of parentage analysis in natural populations. Mol Ecol 12:2511–2523
Jones AG, Small CM, Paczolt KA, Ratterman NL (2010) A practical guide to methods of parentage analysis. Mol Ecol Resour 10:6–30
Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1099–1106
Karron JD, Mitchell RJ (2012) Effects of floral display size on male and female reproductive success in *Mimulus ringens*. Ann Bot 109:563–570
Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. Annu Rev Entomol 28:407–453
Kim S-H, Yi SV (2007) Understanding relationship between sequence and functional evolution in yeast proteins. Genetica 131:151–156
Kim W, Gilet T, Bush JWM (2011) Optimal concentrations in nectar feeding. Proc Natl Acad Sci USA 108:16618–16621
Kingsolver JG, Diamond SE, Siepielski AM, Carlson SM (2012) Synthetic analyses of phenotypic selection in natural populations: lessons, limitations and future directions. Evol Ecol 26:1101–1118
Klein EK, Desassis N, Oddou-Muratorio S (2008) Pollen flow in the wildservice tree, *Sorbus torminalis* (L.) Crantz. IV. Whole interindividual variance of male fecundity estimated jointly with the dispersal kernel. Mol Ecol 17:3323–3336
Klein EK, Carpentier FH, Oddou-Muratorio S (2011) Estimating the variance of male fecundity from genotypes of progeny arrays: evaluation of the Bayesian forward approach. Methods Ecol Evol 2:349–361
Klinkhamer PGL, de Jong TJ, Metz JAJ (1994) Why plants can be too attractive—a discussion of measures to estimate male fitness. J Ecol 82:191–194
Kokubun H, Nakano M, Tsukamoto T, Watanabe H, Hashimoto G, Marchesi E, Bullrich L, Basualdo IL, Kao TH, Ando T (2006) Distribution of self-compatible and self-incompatible populations of *Petunia axillaris* (Solanaceae) outside Uruguay. J Plant Res 119:419–430
Kulbaba MW, Worley AC (2012) Selection on floral design in *Polemonium brandegeei* (Polemoniaceae): female and male fitness under hawkmoth pollination. Evolution 66:1344–1359
Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. Evolution 37:1210–1226
Lankinen A, Hellriegel B, Bernasconi G (2006) Sexual conflict over floral receptivity. Evolution 60:2454–2465
Llaurens V, Castric V, Austerlitz F, Vekemans X (2008) High paternal diversity in the self-incompatible herb *Arabidopsis halleri* despite clonal reproduction and spatially restricted pollen dispersal. Mol Ecol 17:1577–1588

Maloof JE, Inouye DW (2000) Are nectar robbers cheaters or mutualists? Ecology 81:2651–2661
Marshall TC, Slate J, Kruuk LE, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655
Matschiner M, Salzburger W (2009) TANDEM: integrating automated allele binning into genetics and genomics workflows. Bioinformatics 25:1982–1983
Mazer SJ, Delesalle VA, Neal PR (1999) Responses of floral traits to selection on primary sexual investment in *Spergularia marina*: the battle between the sexes. Evolution 53:717–731
Meagher TR (1986) Analysis of paternity within a natural population of *Chamaelirium luteum.* 1. Identification of most-likely male parents. Am Nat 128:199–215
Mitchell RJ (1993) Adaptive significance of *Ipomopsis aggregata* nectar production: observation and experiment in the field. Evolution 47:25–35
Morgan MT, Conner JK (2001) Using genetic markers to directly estimate male selection gradients. Evolution 55:272–281
Nielsen R, Mattila DK, Clapham PJ, Palsbøll PJ (2001) Statistical approaches to paternity analysis in natural populations and applications to the north Atlantic humpback whale. Genetics 157:1673–1682
Nilsson LA (1988) The evolution of flowers with deep corolla tubes. Nature 334:147–149
Oddou-Muratorio S, Klein EK, Austerlitz F (2005) Pollen flow in the wildservice tree, *Sorbus torminalis* (L.) Crantz. II. Pollen dispersal and heterogeneity in mating success inferred from parent-offspring analysis. Mol Ecol 14:4441–4452
Oddou-Muratorio S, Klein EK, Demesure-Musch B, Austerlitz F (2006) Real time patterns if pollen flow in the wild-service tree, *Sorbus torminalis* (Rosaceae). III. Mating patterns and the ecological maternal neighbourhood. Am J Bot 93:1650–1659
Ohashi K, Yahara T (1998) Effects of variation in flower number on pollinator visits in *Cirsium purpuratum* (Asteraceae). Am J Bot 85:219–224
Ohashi K, Yahara T (2001) Behavioural responses of pollinators to variation in floral display size and their influences on the evolution of floral traits. In: Chittka L, Thomson JD (eds) Cognitive ecology of pollination. Cambridge University Press, Cambridge, pp 274–296
Oyama-Okubo N, Ando T, Watanabe N, Marchesi E, Uchida K, Nakayama M (2005) Emission mechanism of floral scent in *Petunia axillaris*. Biosci Biotechnol Biochem 69:773–777
Paccini E, Nepi M (2007) Nectar production and presentation. In: Nicolson SW, Nepi N, Paccini E (eds) Nectaries and nectar. Springer, Dordrecht, pp 167–214
Signorovitch J, Nielsen R (2002) PATRI—paternity inference using genetic data. Bioinformatics 18:341–342
Sink KC (1984) *Petunia*. Monographs on theoretical and applied genetics, vol 9. Springer, Berlin
Smouse PE, Meagher TR (1994) Genetic analysis of male reproductive contributions in *Chamaelirium luteum* (L.) Gray (Liliaceae). Genetics 136:313–322
Smouse PE, Meagher TR, Kobak CJ (1999) Parentage analysis in *Chamaelirium luteum* (L.) Gray (Liliaceae): Why do some males have higher reproductive contributions? J Evol Biol 12:1069–1077
Snow AA, Lewis PO (1993) Reproductive traits and male fertility in plants: empirical approaches. Annu Rev Ecol Syst 24:331–351
Stinchcombe JR, Agrawal AF, Hohenlohe PA, Arnold SJ, Blows MW (2008) Estimating nonlinear selection gradients using quadratic coefficients: Double or nothing? Evolution 62:2435–2440
Stuurman J, Hoballah ME, Broger L, Moore J, Basten C, Kuhlemeier C (2004) Dissection of floral pollination syndromes in *Petunia*. Genetics 168:1585–1599
R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/
Teixeira S, Burkhardt A, Bernasconi G (2008) Genetic variation among females affects paternity in a dioecious plant. Oikos 117:1594–1600
Tsukamoto T, Ando T, Takahashi K, Omori T, Watanabe H, Kokubun H, Marchesi E, Kao TH (2003) Breakdown of self-incompatibility in a natural population of *Petunia axillaris* caused by loss of pollen function. Plant Physiol 131:1903–1912
van Kleunen M, Burczyk J (2008) Selection on floral traits through male fertility in a natural plant population. Evol Ecol 22:39–54
van Kleunen M, Ritland K (2004) Predicting evolution of floral traits associated with mating system in a natural plant population. J Evol Biol 17:1389–1399
Venail J, Dell'Olivo A, Kuhlemeier C (2010) Speciation genes in the genus *Petunia*. Philos Trans R Soc B 365:461–468
Wright JW, Meagher TR (2004) Selection on floral characters in natural Spanish populations of *Silene latifolia*. J Evol Biol 17:382–395